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EXTRACTS OF *Flourensia cernua* (L): VOLATILE CONSTITUENTS AND ANTIFUNGAL, ANTIALGAL, AND ANTITERMITE BIOACTIVITIES[1]

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Abstract—The chemical components of tarbush (*Flourensia cernua*) leaves were fractionated by extracting successively with hexanes, diethyl ether, and ethanol. Volatile profiles of each fraction were identified by using GC-MS. The hexanes fraction contained mostly monoterpenoids, while the ethanol fraction volatiles were primarily sesquiterpenoids. Crude fractions were tested for activity against fungi, algae, and termites. Application of as little as 1 μg of the essential oil from the hexanes fraction was sufficient to provide visible antifungal activity in bioautography assays. The diethyl ether fraction showed selective activity against the cyanobacterium responsible for the 2-methylisoborneol-induced off-flavor sometimes associated with catfish farming operations. All three fractions exhibited a high degree of antitermite activity.

Key Words—*Flourensia cernua*, fractionation, activity, fungi, cyanobacteria, algae, termite, *Colletotrichum*, *Oscillatoria*, *Selenastrum*, *Reticulitermes*.

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INTRODUCTION

At the Natural Products Utilization Research Unit (NPURU), Oxford, Mississippi, we are interested in finding novel uses for products from natural sources, especially as they relate to pest management. *Flourensia cernua* DC (tarbush, hojasen) is an aromatic deciduous shrub endemic to the Chihuahuan Desert of the southwestern United States and northern Mexico. Tarbush flowers are reported to be acutely toxic to livestock (Mathews, 1944; Dollahite and Allen, 1975) when consumed in large amounts (approximately 1% of body weight per day). Additionally, leaves and flower heads are sold in drug markets in Mexico (Dillon, 1984) and local farmers' markets in the United States as a remedy for indigestion. Tarbush is increasing in dominance within the Chihuahuan Desert and is currently the target of several studies involving interactions of herbivory and shrub chemistry at the Jornada Experimental Range (JER), Las Cruces, New Mexico. We previously reported the chemical profile of the essential oil of *F. cernua* (Tellez et al., 1997). In collaboration with the JER, we examined a set of crude extracts obtained from tarbush leaves by sequential extractions with solvents of widely differing properties (hexanes, ether, and ethanol). Bioassays conducted at the JER show all three crude fractions deter consumption by sheep when applied to alfalfa pellets (Estell et al., 2001). Bioassays conducted at the NPURU have shown that the crude hexanes and ether extracts are phytotoxic, whereas the ethanol fraction is inactive (Dayan and Tellez, 1999). Little else is known of the bioactivity of tarbush constituents.

Because volatile chemicals are likely the first line of exposure of a plant to herbivores, one objective of this research was to examine volatile chemical profiles of the three fractions that were deterrent to consumption by sheep (Estell et al., 2001). A second objective was to test these fractions for antifungal, antialgal, and antitermitic activity in an effort to identify potential agricultural and pest management uses of material from this invasive shrub.

METHODS AND MATERIALS

Plant Collection. Plant materials were collected from an area heavily infested with tarbush on the JER. Approximately 300 plants were selected haphazardly over a large area. Leaves were stripped from leaders into plastic bags by hand using gloves, and immediately placed on ice. Leaves were frozen within 2 hr, shipped on Dry Ice to the NPURU, and stored at -20°C until extraction.

Extractions. Tarbush leaves (36.5 kg) were extracted at room temperature in approximately 3-kg batches. Extractions were carried out sequentially with 7 liters each of hexanes, diethyl ether, and 100% ethanol for 22 hr in covered 10-liter round bottom flasks placed in a circular shaker (New Brunswick Co. G10 Gyrotory

Shaker) at 150 rpm. Each fraction was filtered (Whatman No. 1) and the solvents removed under reduced pressure with a rotary evaporator. All fractions from a given solvent were combined. The hexanes fraction produced 415.5 g of a highly aromatic yellow–brown oil, the ether fraction resulted in 984.0 g of a thick green oil, and the ethanol fraction produced 2215.6 g of an extremely thick green oil. All final products were stored at -20°C until used for bioassays. Steam distillations of the extracts were conducted as previously described (Tellez et al., 2000).

Chemical Analyses. Volatile chemical analyses of extracts were performed by GC-MS (EI, 70 eV) with a DB-5 column (30 m $\times$ 0.25 mm fused silica capillary column, film thickness 0.25 μm) with He as carrier gas (1 ml/min), 1 μl injection size, and a programmed (injector temperature: 220°C, transfer line temperature: 240°C, initial column temperature: 60°C, final column temperature: 240°C, 3°C/min) run (Adams, 1995). Octane and eicosane standards were used only for determination of retention times. Identification of extract components was performed by a comparison of mass spectra with literature data (NIST/EPA/NIH, 1990; Adams, 1995), and by comparison of their relative retention times with those of authentic compounds, or by comparison of their retention indices with those in the literature (Adams, 1995). The relative amounts (RA) of individual components of the extracts are expressed as percent peak area relative to total peak area. Analyses were performed on extracts after removal of solvents, since this was representative of their composition as applied to bioassays.

Algicidal Assays. A rapid bioassay (Schrader et al., 1997) was used to determine the lowest-observed-effect concentration and the lowest-complete-inhibition concentration of *F. cernua* extracts towards isolates of the cyanobacteria *Oscillatoria perornata* and *Oscillatoria agardhii,* and the green alga *Selenastrum capricornutum*. Ether, ethanol, and hexanes extracts were dissolved in technical grade acetone, ethanol, and hexane, respectively, and placed into microplate wells by the method of Schrader et al. (1997) to achieve a final test concentration of 50 μg/ml ($N = 4$). Solvents were allowed to evaporate for 10–15 min before adding culture material. Solvents without extracts were used in control microplate wells. The bioassay was modified by using 96-well quartz microplates (Hellma Cells, Inc., Forest Hills, New York) because hexane and acetone were used as loading solvents and are not compatible with polystyrene microplates.

Fungicidal Assays. Pathogen production and inoculum preparation for *Colletotrichum fragariae* Brooks, *C. gloeosporioides* Penz. & Sacc., and *C. accutatum* Simmonds were performed according to published procedures (Wedge and Kuhajek, 1998). Conidia concentrations were determined photometrically (Espinel-Ingroff and Kerkering, 1991; Wedge and Kuhajek, 1998) from a standard curve, and suspensions were adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/ml. Inhibition of fungal growth on chromatographic plates was evaluated by modifications of thin-layer chromotography (TLC) bioautographic assays (Homans and Fuchs, 1970).

Extracts were spotted on a TLC plate with a disposable glass micropipet, loading 400, 100, 10, 1, and 0.1 μg of each extract and of each essential oil derived from each extract. Loading solvents were used as controls. Each experiment was repeated three times. To detect biological activity directly on the TLC plate, silica gel plates (250 μm, Silica Gel GF Uniplate, Analtech, Inc., Newark, Delaware) were sprayed with a spore suspension as previously described (Wedge and Nagle, 2000). Inhibition of fungal growth for each test fungus was measured four days after treatment. Concentration-dependent sensitivity of the fungal species to each extract or oil was determined by comparing size of inhibitory zones.

Termite Assays. Extracts were tested at different concentrations, proportional to extract recovery. Hexanes, ether, and ethanol extracts (189, 405, and 958 mg, respectively) were each suspended with vigorous shaking in 45 ml of acetone. A 3-ml aliquot of the suspension was placed on each of three absorbent cellulose pads (47 mm diam.) for each of the three extracts ($N = 9$). Pads of cellulose without extract ($N = 3$) were used as a control. All pads remained under a fume hood for 24 hr to allow evaporation of acetone. Plastic containers (5 cm diam. $\times$ 3.5 cm high) served as test containers. A sterile sand–vermiculite (1:1) mixture (50 g) moistened with distilled water was placed in each of 12 test containers. One hundred *Reticulitermes* sp. worker termites were placed on top of the sand in each test unit. After 10 min, a treated cellulose pad that had been moistened with distilled water was placed in each container. Each container was covered and placed in an incubator maintained at 25°C and 53% relative humidity. All test units were observed weekly for five weeks to note any dead termites or unusual termite behavior. After five weeks, test units were disassembled, and termite survival and amount of feeding on treated and untreated cellulose pads was recorded.

RESULTS AND DISCUSSION

Table 1 shows the identity, retention index, retention time, and percent composition of the volatile compounds in the hexanes, ether, and ethanol extracts. The volatile profiles for the three crude fractions were markedly different. Forty-one volatile compounds were identified in the hexanes fraction, accounting for over 91.3% of the composition of the volatiles in the extract. Forty-seven volatile compounds were identified in the ether fraction, accounting for over 84.7% of the composition of the volatiles in the extract; δ-selinene (5.8%) was tentatively identified by MS only. Fourteen volatile compounds were identified in the ethanol fraction, accounting for over 79.3% of the composition of the volatiles in the extract; δ-selinene (8.7%) and 3,7,11,15-tetramethyl-2-hexadecene-1-ol (4.5%) were tentatively identified by MS only. The volatiles in the hexanes extract consisted of a high proportion of monoterpenes, with the 27 identified monoterpenes accounting for 74.7% of the total area. The hexanes fraction contained a low proportion

TABLE 1. VOLATILE CONSTITUENTS OF HEXANES, ETHER, AND ETHANOL SEQUENTIAL EXTRACTIONS OF TARBUSH LEAVES[a]

			RA		
	R_t (min)	RI	Hexanes	Ether	Ethanol
santolina triene	282	907	t	—	—
tricyclene	306	927	t	—	—
α-thujene	311	931	0.1	0.1	—
α-pinene	322	939	2.0	1.4	—
camphene	345	954	0.9	0.1	—
sabinene	383	977	1.9	0.4	—
β-pinene	390	980	0.8	0.3	—
myrcene	413	992	19.9	6.7	—
α-phellandrene	436	1004	0.1	t	—
3-δ-carene	449	1012	12.6	6.1	—
α-terpinene	460	1018	t	t	—
o-cymene	470	1023	t	—	—
p-cymene	474	1026	0.1	t	—
limonene	486	1032	27.7	8.4	—
1,8-cineole	495	1033	t	t	—
z-β-ocimene	500	1040	t	—	—
e-β-ocimene	522	1050	0.4	0.1	—
γ-terpinene	545	1061	t	t	—
artemisia ketone	547	1062	t	—	—
artemisia alcohol	598	1084	4.3	0.6	—
p-mentha-2,4(8)diene	604	1086	t	t	—
terpinolene	610	1089	0.1	t	—
trans-pinocarveol	723	1139	t	—	—
camphor	736	1145	0.1	—	—
cis-chrysanthenol	782	1163	1.1	0.2	—
borneol	789	1166	2.4	0.5	0.2
terpin-4-ol	817	1177	t	—	—
δ-elemene	1222	1340	t	0.4	0.9
α-cubebene	1251	1352	—	0.2	—
cyclosativene	1292	1367	—	0.1	—
α-ylangene	1305	1372	—	0.1	—
α-copaene	1316	1376	t	0.2	—
β-bourbonene	1337	1384	t	t	—
β-cubebene	1351	1389	—	0.2	—
cis-jasmone	1366	1394	0.1	—	—
α-cedrene	1406	1409	—	0.2	0.2
β-caryophyllene	1421	1417	0.9	16.0	14.3
trans-α-bergamotene	1463	1435	—	0.1	—
α-guaiene or aromadendrene	1468	1437	—	t	—
α-humulene	1503	1452	0.4	5.0	6.9
γ-muurolene	1561	1476	—	1.0	0.1
γ-curcumene	1563	1477	—	t	—
germacrene D	1569	1479	1.6	24.0	43.3

TABLE 1. CONTINUED

	R_t (min)	RI	RA		
			Hexanes	Ether	Ethanol
β-selinene	1581	1484	—	0.9	1.1
viridiflorene	1595	1489	—	t	—
cis-β-guaiene	1601	1492	0.1	—	—
bicyclogermacrene	1606	1494	0.1	1.1	1.6
δ-selinene (MS only)[b]	1633	1505	0.3	5.8	8.7
trans-γ-cadinene	1650	1513	0.1	0.9	0.6
δ-cadinene	1670	1522	0.1	1.2	0.9
cadina-1,4-diene	1689	1531	—	0.1	—
α-cadinene	1702	1537	—	0.1	—
germacrene B	1745	1555	—	0.2	0.3
caryophyllene oxide	1803	1580	0.2	0.1	0.2
β-eudesmol	1956	1649	11.3	2.8	4.4
flourensadiol	2403	1864	1.4	4.6	4.5
3,7,11,15-teramethyl-2-hexadecene-1-ol (MS only)	2873	2114	—	—	4.5

[a] RT = retention time; RI = retention index as determined on DB-5 using the homologous series of *n*-hydrocarbons; RA = relative area (peak area relative to total peak area); t = trace (<0.05%); — = not found.
[b] Tentative identification (by MS only).

of sesquiterpenoids, with β-eudesmol accounting for 11.3%, and the remaining 13 accounting for 4.9% of the total relative area. The major monoterpenoids were myrcene (19.9%), 3-δ-carene (12.6%), and limonene (27.7%). The identified volatile compounds in the ether extract consisted of a mixture of monoterpenes (24.9%) and sesquiterpenes (57.0%), with a marked decrease in the proportion of β-eudesmol present (2.8%). The major components included myrcene (6.7%), 3-δ-carene (6.1%), limonene (8.4%), β-caryophyllene (16.0%), α-humulene (5.0%), and germacrene D (24.0%). Finally, the ethanol extract volatile profile was almost completely devoid of monoterpenes (0.2%) and consisted almost exclusively of sesquiterpenes, with the identified sesquiterpenes accounting for 79.1% of the total area. The major constituents included β-caryophyllene (14.3%), α-humulene (6.9%), and germacrene D (43.3%).

The three solvents used to extract tarbush leaves differed in polarity, from the fairly nonpolar hexanes to the fairly polar ethanol. These solvents were selected in order to obtain a crude separation of the components of the leaves. Although selective extraction was achieved, the separation appeared more a function of size (monoterpenoids versus sesquiterpenoids) than polarity, at least for volatile compounds. The observed difference is not an artifact of solvent removal since chromatograms obtained prior to and after solvent removal showed little difference in profile. In the past, we have observed selective extractions of terpenoid

classes with *Artemisia annua* (Tellez et al., 1999; Duke et al., 2000), although what mechanisms might be responsible in this present case remain unexplained.

Steam distillations of each extract afforded 337, 178, and 15 mg of essential oil (volatiles) per gram of hexanes, ether, and ethanol extract, respectively. Thus, a substantial portion of all three crude extracts represented the nonvolatile fraction. This distribution of volatile and nonvolatile components among the three extracts might explain results obtained in sheep feeding studies (Estell et al., 2001), in which all extracts decreased feed intake even though the amounts and profiles of the volatiles were different.

This study demonstrated that all three crude fractions were active in our fungal bioassays. Bioautography data (Table 2) showed that the essential oil derived from the hexanes extract retained activity against *C. gloeosporioides* and *C. fragariae* at lower application amounts than the other treatments. Antifungal activity of the essential oil derived from the hexanes crude extract was detected at the 1 μg dose. Antifungal activity of the essential oils derived from the ether and ethanol fractions was observed only at 10 μg doses and above. Antifungal activity of the ethanol crude extract fraction could be observed only at the highest amount (400 μg)

TABLE 2. FUNGAL GROWTH INHIBITION BY HEXANES, ETHER, AND ETHANOL EXTRACTS OF TARBUSH LEAVES AND THEIR ESSENTIAL OILS BY BIOAUTOGRAPHY USING *Colletotrichum gloeosporioides*, *C. fragaria*, AND *C. accutatum* AS INDICATORS

	Inhibition zone diameter (mean ± SD, mm)					
	Hexanes		Ether		Ethanol	
	Extract	Oil	Extract	Oil	Extract	Oil
C. gloeosporioides						
400 μg	7.3 ± 1.2	8.7 ± 0.9	10.0 ± 0.8	9.3 ± 0.5	9.7 ± 0.5	13.7 ± 0.9
100 μg	3.7 ± 0.5	6.3 ± 1.2	6.7 ± 0.5	8.3 ± 0.9	0.0 ± 0.0	9.3 ± 0.9
10 μg	0.0 ± 0.0	3.0 ± 2.2	1.0 ± 1.4	2.7 ± 0.5	0.0 ± 0.0	3.3 ± 0.5
1 μg	0.0 ± 0.0	0.7 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C. fragariae						
400 μg	10.3 ± 1.2	11.0 ± 0.8	10.3 ± 1.2	11.3 ± 3.3	10.0 ± 0.8	12.7 ± 0.5
100 μg	8.0 ± 0.8	8.3 ± 1.2	7.3 ± 0.5	8.7 ± 0.9	0.0 ± 0.0	11.7 ± 0.9
10 μg	2.3 ± 0.5	3.7 ± 0.9	1.3 ± 1.9	3.3 ± 0.5	0.0 ± 0.0	6.7 ± 3.1
1 μg	0.0 ± 0.0	2.3 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C. accutatum						
400 μg	7.0 ± 0.8	8.3 ± 0.5	10.7 ± 0.5	7.7 ± 0.5	5.3 ± 0.5	10.3 ± 0.5
100 μg	4.7 ± 0.5	5.7 ± 0.5	6.7 ± 0.5	6.3 ± 0.5	0.0 ± 0.0	9.0 ± 0.8
10 μg	0.0 ± 0.0	2.0 ± 1.4	1.0 ± 1.4	2.3 ± 1.7	0.0 ± 0.0	4.7 ± 0.9
1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

tested at the end of four days. Comparison among extracts, other than comparing the presence or absence of activity, by comparing size of inhibition zones is not valid because of the different solvents used to spot each extract. Comparing the zones of inhibition within an extract indicates a clear dose response for all three extracts against *C. gloeosporioides*, *C. fragariae*, and *C. accutatum*.

The essential oil of the hexanes extract was consistently more active against fungi than the extract itself, suggesting that a good portion of the activity in the hexanes extract resides in its steam-distilled volatile components. Several major volatile components—α-pinene (2.0%), 3-δ-carene (12.6%), and limonene (27.7%) (Himejima et al., 1992), and β-eudesmol (11.3%) (Miyakado et al., 1976)—are reported to have antifungal activity. Another major component, myrcene (19.9%), is reported to enhance the activity of other antimicrobials (Onawunmi et al., 1984) . The steam-distilled volatiles of the ethanol fraction exhibited inhibitory activity to all three *Colletotrichum* species, but their extremely small presence (1.5%) in the extract makes it unclear whether the inhibitory effect observed for the extract is due mostly to these steam-distilled volatiles or to other non-steam-distilled components.

F. cernua extracts were screened against two species of cyanobacteria (blue-green algae) and one species of green algae to determine their potential as a selective cyanobactericide. An off-flavor in channel catfish (*Ictalurus punctatus*) raised in the southeastern United States creates an unpalatable and, therefore, unmarketable product that results in large economic losses to the industry. Most off-flavor episodes in catfish are attributed to their absorption of earthy/musty compounds produced by certain species of cyanobacteria that grow in catfish production ponds. In west Mississippi, the cyanobacterium *Oscillatoria perornata* (Skuja), a producer of the musty-odor compound 2-methylisoborneol (MIB), is thought to be the major cause of musty off-flavor in farm-raised catfish (van der Ploeg et al., 1995). Green algae are not associated with such undesirable metabolites and are also preferable to cyanobacteria in catfish production ponds because they are better oxygenators of the water and a better base for aquatic food chains (Paerl and Tucker, 1995). Therefore, the discovery of safe compounds that selectively kill cyanobacteria would benefit the channel catfish industry.

Complete inhibition of *O. perornata* was observed for the hexanes and ether extracts of *F. cernua* at 50 (μg/ml (Figure 1B). For *Oscillatoria agardhii* (a non-MIB-producing cyanobacterium), complete inhibition occurred with the hexanes extract, and these results were established visually, i.e., disappearance of filaments in the treatment wells. The formation of black precipitate in wells containing hexanes extracts and *O. agardhii* culture resulted in high absorbance readings that could be misinterpreted as accelerated growth rate by *O. agardhii* based solely on the graphed data (Figure 1A). The other two cultures did not produce any colored precipitate in wells containing hexanes extract. For *S. capricornutum*, complete inhibition was also established only for the hexanes extract (Figure 1C).

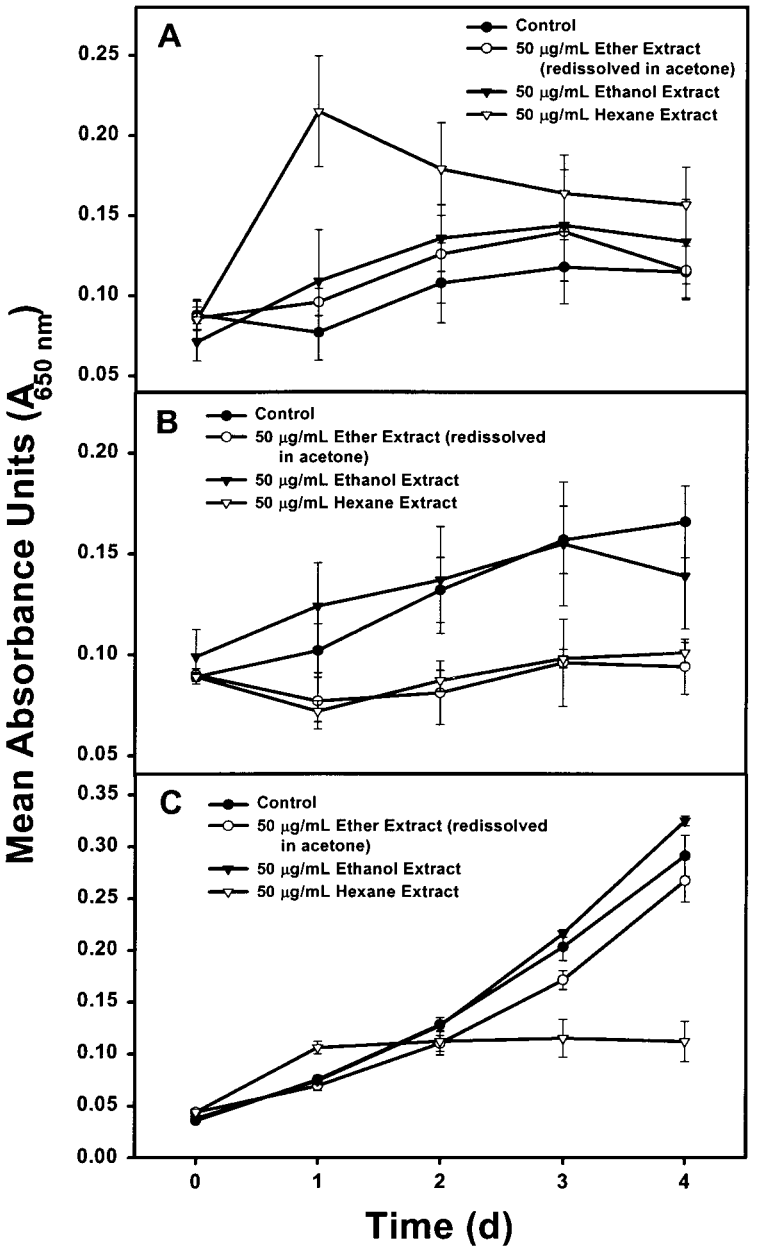

FIG. 1. Effect of tarbush extracts on growth of (**A**) *Oscillatoria agardhii*, (**B**) *O. perornata*, and (**C**) *Selenastrum capricornutum*. Error bars represent standard deviations of the mean ($n = 3$).

These results indicate selective toxicity of the ether extract of *F. cernua* towards *O. perornata* and nonselective toxicity of the hexanes extract towards all three organisms. No toxicity towards the green alga or cyanobacteria tested was observed for the ethanol extract. Additional screening of extracts and their steam-distilled oils revealed that a constituent (s) present in the extract but not the steam-distilled oil is (are) responsible for the selective toxicity of the ether extract towards *O. perornata* (data not shown).

All three crude extracts also showed a high degree of termiticidal activity after five weeks. The ethanol fraction was tested at the highest concentration and had a mean survival of 0%; there were no survivors in any of three replicates ($N = 100$ per replicate). The hexanes extract was tested at the lowest concentration but also had a nearly complete kill (mean of 2.3% survival); there were no survivors in two of the replicates and only seven survivors on the third replicate ($N = 100$ per replicate). The intermediate ether fraction had 15.7% survival (0, 35, and 12 survivors in each replicate, $N = 100$/replicate). Survival of the control group with an acetone blank was 82%. The antitermite properties of different fractions suggest the presence of more than one active compound (or set of compounds).

In conclusion, the three sequential extracts of tarbush exhibited fairly different volatile profiles. The crude organic extracts of tarbush show promise as antitermitic and antifungal agents. The ether extract of *F. cernua* holds promise as a possible selective cyanobactericide against the cyanobacterium thought to be the major cause of musty off-flavor problems in Mississippi farm-raised catfish. We are currently carrying out the bioassay-guided isolation and identification of the active components in *F. cernua.*

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